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Sprouty1 Is a Critical Regulator of GDNF/RET-Mediated Kidney Induction

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Summary

Intercellular signaling molecules and their receptors, whose expression must be tightly regulated in time and space, coordinate organogenesis. Regulators of intracellular signaling pathways provide an additional level of control. Here we report that loss of the receptor tyrosine kinase (RTK) antagonist, Sprouty1 *(Spry1)*, causes defects in kidney development in mice. *Spry1*$^{-/-}$ embryos have supernumerary ureteric buds, resulting in the development of multiple ureters and multiplex kidneys. These defects are due to increased sensitivity of the Wolffian duct to GDNF/RET signaling, and reducing *Gdnf* gene dosage correspondingly rescues the *Spry1* null phenotype. We conclude that the function of *Spry1* is to modulate GDNF/RET signaling in the Wolffian duct, ensuring that kidney induction is restricted to a single site. These results demonstrate the importance of negative feedback regulation of RTK signaling during kidney induction and suggest that failures in feedback control may underlie some human congenital kidney malformations.

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Introduction

It has become clear that many steps in mammalian development depend not only on mediators of intercellular signaling but also on molecules that negatively regulate this signaling. For example, mutations in genes encoding antagonists of the TGFβ and WNT pathways, which play major roles in mammalian embryogenesis, often result in developmental defects (Bachiller et al., 2000; Matzuk et al., 1995; Mukhopadhyay et al., 2001; Perea-Gomez et al., 2002; Zuniga et al., 1999). There is accumulating evidence that inhibitors of receptor tyrosine kinase (RTK) signaling also play important roles during organogenesis (Freeman, 2000).

Studies in *Drosophila* identified the *sprouty (spry)* gene as a negative feedback regulator of branching morphogenesis of the tracheal system (Hacohen et al., 1998), compound eye development (Casci et al., 1999), and other developmental processes (Kramer et al., 1999; Reich et al., 1999) mediated by Fibroblast Growth Factor (FGF) and other RTK signaling pathways. Four vertebrate orthologs of the *Drosophila spry* gene have been identified. Both loss- and gain-of-function experiments in mouse (Chi et al., 2004; Minowada et al., 1999), chicken (Chambers et al., 2000), zebrafish (Furthauer et al., 2001), and *Xenopus* (Nutt et al., 2001) embryos indicate that RTK signaling, at least via the FGF pathway, is both necessary and sufficient for Sprouty gene expression.

Misexpression or overexpression studies of Sprouty genes in zebrafish (Furthauer et al., 2001), frog (Nutt et al., 2001), chick (Minowada et al., 1999), and mouse (Chi et al., 2004; Lee et al., 2001; Mailleux et al., 2001; Perl et al., 2003; Tefft et al., 1999) embryos or tissues suggested that these genes play important roles in vertebrate development. However, there have been few loss-of-function experiments to directly test this hypothesis, including gene knockdown studies in zebrafish embryos (Furthauer et al., 2001) and mouse lung and kidney explants (Gross et al., 2003; Tefft et al., 1999). Here we describe the production of a *Spry1* null allele in the mouse and demonstrate that *Spry1* is essential for normal kidney development.

The first morphologically discernable step of kidney development in the mouse is the outgrowth of the ureteric bud (UB) from the caudal Wolffian duct at E11 (Saxen, 1987). Formation of the UB, which develops into the collecting system of the kidney, is induced by glial cell line-derived neurotrophic factor (GDNF). GDNF is produced by the metanephric mesenchyme and activates a RTK (RET)/coreceptor (GFRα1) complex on the Wolffian duct epithelium, resulting in UB outgrowth (Vainio and Lin, 2002). Accordingly, *Gdnf*, *Ret*, or *Gfrα1* null embryos exhibit renal agenesis due to failure of UB formation (Cacalano et al., 1998; Moore et al., 1996; Pachnis et al., 1993; Pichel et al., 1996; Sanchez et al., 1996).

Gdnf expression is under tight spatiotemporal control during development. Prior to kidney induction, *Gdnf* is expressed along the anterior-posterior length of the

nephrogenic mesenchyme. By the time that the UB forms (40 somite stage), *Gdnf* expression is restricted to the posterior mesenchyme adjacent to the presumptive site of UB outgrowth (Grieshammer et al., 2004). Mutations that allow *Gdnf* expression to be maintained in the anterior nephrogenic mesenchyme result in supernumerary UBs and the formation of multiplex kidneys with multiple abnormal ureters that often fail to connect properly to the bladder (Grieshammer et al., 2004; Kume et al., 2000). This condition resembles the human syndrome of congenital anomalies of the kidney and urinary tract (CAKUT) that occur in about 1/200 pregnancies (Ichikawa et al., 2002).

In the present study, we show that *Spry1* inhibits the responsiveness of the Wolffian duct to GDNF signaling, thus ensuring the formation of a single UB during induction of the metanephric kidney.

Results

Spry1$^{-/-}$ Mice Exhibit Kidney and Urinary Tract Anomalies

Using Cre/lox technology, we produced mouse lines carrying a conditional (*Spry1*^{flox}) allele in which the *Spry1* open reading frame was flanked with *loxP* sites and a null (*Spry1*$^{-}$) allele in which the entire coding sequence was deleted (Figure 1). Mice homozygous for the *Spry1*^{flox} allele or heterozygous for the null allele (*Spry1*$^{+/-}$) were viable, fertile, and appeared phenotypically normal.

Homozygous *Spry1*$^{-/-}$ mice were born at the expected Mendelian ratios, but viability after birth was reduced. Of 38 pups born, 8 (21%) died within 48 hr and 27 (71%) died within 5 months. Of 21 *Spry1*$^{-/-}$ animals examined shortly after birth, 20 displayed uni- or bilateral ureter (multiple ureters, hydroureter) and kidney malformations (Figure 2). Abnormal ureters ended blindly in females (Figures 2A and 2B) and were attached to the vas deferens in males (Figures 2C–2F). Histological analyses revealed disorganized kidneys in which the nephrogenic zones were not restricted to the cortex as in normal newborn kidneys (Figure 2G) but extended throughout the kidney (Figure 2H), suggesting that what appeared macroscopically as a single unit was actually several kidney primordia fused together. The mutant kidneys frequently contained multiple epithelial cysts (Figure 2H) that stained strongly with dolichus biflorus agglutinin (DBA) lectin (Figure 2I), indicating that they were derived from the collecting system of the kidney (Qiao et al., 1995). These phenotypes are characteristic of human CAKUT (Ichikawa et al., 2002). Although the genetic causes of CAKUT are not known, abnormalities during the initial phases of kidney induction were suggested as the likely underlying cause (Ichikawa et al., 2002). We therefore analyzed early kidney development in *Spry1*$^{-/-}$ embryos.

Urinary Tract Defects in *Spry1*$^{-/-}$ Animals Result from Supernumerary Ureteric Bud Formation

We assessed Wolffian duct and UB morphology in E11.0–E11.5 embryos by in situ hybridization for *Ret* (Kume et al., 2000; Pachnis et al., 1993). *Spry1*$^{-/-}$ embryos exhibited abnormally wide UBs compared to stage-matched controls at E11.0 (Figures 3A and 3B), and supernumerary buds were evident along the mutant duct (Figure 3B). By E11.5, the normal UB in *Spry1*$^{+/-}$ embryos demonstrated two branched tips connected by a narrow stalk to the Wolffian duct at the level of the posterior edge of the hindlimb bud (Figure 3C). In contrast, the UB that formed at the normal position in *Spry1*$^{-/-}$ embryos had an abnormally wide stalk and tips (Figure 3D). In addition, ectopic UBs were observed anterior to this wide UB (Figure 3D).

To visualize subsequent branching morphogenesis of the UBs, E11.5 kidney rudiments were cultured in vitro. *Spry1*$^{+/-}$ UBs underwent regular, reiterative branching to give rise to a characteristic ureteric tree (Figure 3E). The UB tips induced condensation of the metanephric mesenchyme, indicated by PAX2-positive cells surrounding the bud tips (Figure 3E). By contrast, some mutant kidneys demonstrated multiple ureteric stalks attached to the Wolffian duct, each of which had undergone branching morphogenesis that resulted in irregular, wide, and occasionally fused UB tips (Figure 3F). PAX2 expression was evident around most of the UB tips in *Spry1*$^{-/-}$ explants (Figure 3F), indicating that although the architecture of *Spry1*$^{-/-}$ UBs was abnormal, they qualitatively retained the capacity to initiate differentiation of the metanephric mesenchyme.

Between E12.5 and E14.5, normal developing ureters undergo a maturation process during which the base of the ureter disconnects from the Wolffian duct and inserts into the bladder (Batourina et al., 2002). In E14.5 *Spry1*$^{-/-}$ embryos, the supernumerary, abnormal ureters remained attached to the Wolffian duct (Figures 3G and 3H). Since the anterior portion of the Wolffian duct develops into the vas deferens in male embryos and degenerates in female embryos, these observations explain the ureter anomalies in *Spry1*$^{-/-}$ newborn animals (Figure 2).

Spry1 Expression in the Wolffian Duct Is Regulated by GDNF/RET Signaling

To understand how *Spry1* regulates UB formation, we examined *Spry1* expression at the onset of kidney development. At E11.0, *Spry1* was detected along the length of the Wolffian duct with highest levels in the posterior duct, where the UB forms. *Spry1* RNA was also detected in the nephrogenic mesenchyme, albeit at a significantly lower level (Figure 4A). Since the expression of Sprouty genes is generally induced by the signaling pathways they inhibit (Chambers et al., 2000; Hacohen et al., 1998; Mailleux et al., 2001; Minowada et al., 1999; Nutt et al., 2001; Ozaki et al., 2001; Reich et al., 1999), and UB formation is induced by the activation of RET by GDNF (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), we asked whether *Spry1* expression is dependent upon RET activity. *Spry1* expression was significantly reduced in *Ret*$^{-/-}$ Wolffian ducts (Figure 4B), and beads soaked in recombinant GDNF and implanted into intermediate mesoderm explants rapidly upregulated *Spry1* gene expression in the Wolffian duct (Figures 4C and 4D).

Spry1 Is Not Necessary for Normal Spatiotemporal Control of *Gdnf* Expression but Is Required in the Wolffian Duct to Regulate Ureteric Bud Formation

Analysis of other mouse mutants had indicated that supernumerary UBs can develop due to the abnormal

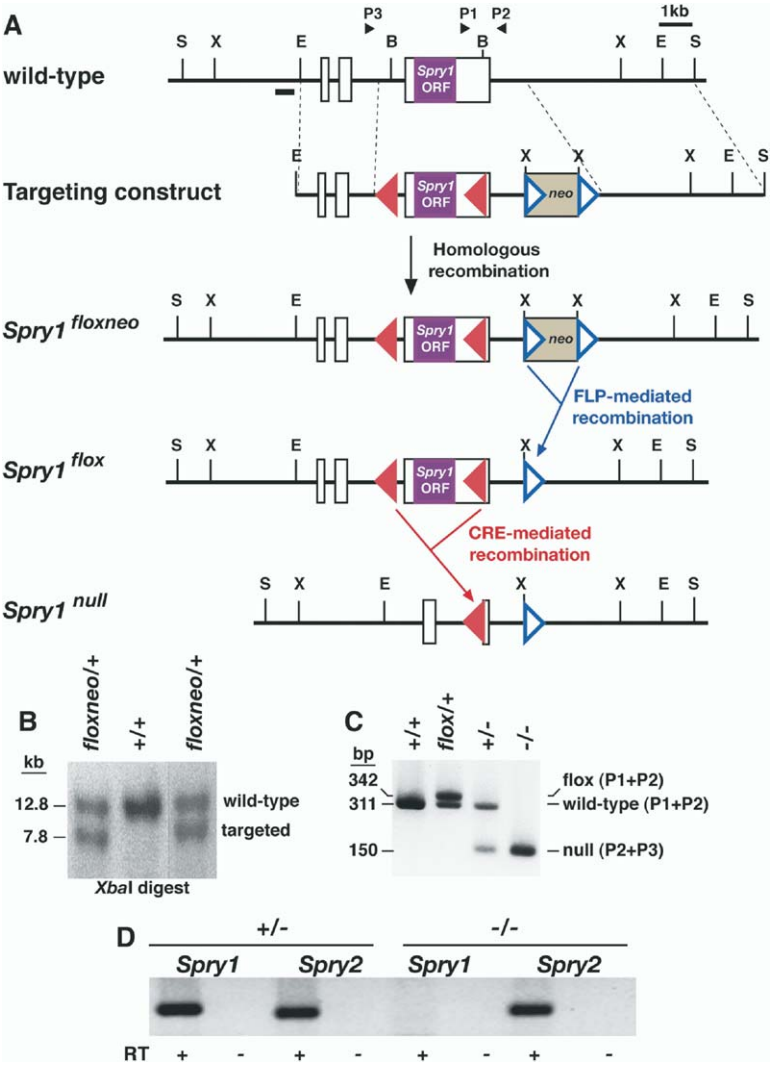

Figure 1. Generation of Conditional and Null *Spry1* Alleles in Mice

(A) Schematic representation of the *Spry1* wild-type allele, the targeting construct, and the *Spry1*^{floxneo} allele produced by homologous recombination at the *Spry1* locus in ES cells. A horizontal line represents *Spry1* genomic DNA; the purple box represents the *Spry1* open reading frame (ORF), which is contained within one of the three exons (open boxes). The complete *Spry1* ORF was flanked with *loxP* sites (red triangles), and a PGKneo cassette flanked by *frt* sites (open blue triangles) was introduced 3′ of the gene. The PGKneo cassette was removed by breeding mice carrying *Spry1*^{floxneo} with *β-Actin/Flpe* deleter mice (Rodriguez et al., 2000) to generate a *Spry1*^{flox} allele. *Spry* $^{flox/+}$ animals were bred with *β-Actin/Cre* deleter mice to produce animals with *Spry1* null allele. B, *Bgl*II; E, *EcoR*I; S, *Spe*I; X, *Xba*I.

(B) ES cell clones containing the targeted allele (*Spry1*^{floxneo}) were identified by Southern blot using a probe containing sequences 5′ of those used in the targeting vector (black bar). Results are shown for two independent clones heterozygous for the targeted allele and a wild-type clone.

(C) Mice were genotyped by PCR using primers (P1, P2, P3) located at the positions shown above the diagram of the wild-type allele.

(D) RT-PCR assay of RNA extracted from newborn *Spry1*$^{+/-}$ and *Spry1*$^{-/-}$ kidneys using primer pairs specific for *Spry1* or *Spry2* coding sequences. Note that *Spry1*-encoding RNA is not detected in *Spry1*$^{-/-}$ mice, whereas *Spry2*-encoding RNA is present. + or –RT indicates the presence or absence of reverse transcriptase in the reaction as a control for genomic DNA contamination.

persistence of *Gdnf* expression in the anterior mesenchyme (Grieshammer et al., 2004; Kume et al., 2000). However, careful examination of *Spry1*$^{-/-}$ embryos at the time of bud induction revealed no such anterior maintenance of *Gdnf* expression (Figures 4E and 4F). Therefore, a more likely explanation for the UB defects in *Spry1*$^{-/-}$ animals is that *Spry1* functions in the Wolffian duct to regulate signaling downstream of RET. To determine whether *Spry1* acts primarily in the Wolffian duct during kidney development, we specifically deleted *Spry1* in the duct by crossing mice carrying a conditional allele of *Spry1* with a *Hoxb7/Cre* transgenic line that expresses *cre* in the duct but not the mesenchyme from E9.5 onward (Yu et al., 2002). In situ hybridization showed that *Spry1* expression was absent from the UB epithelium of *Hoxb7/Cre;Spry1*$^{flox/flox}$ embryos at E11.5 but was still detectable in the mesenchyme (Figures 4H and 4I). *Spry1* RNA was not detectable in either tissue in *Spry1*$^{-/-}$ embryos (Figure 4J). The kidneys of newborn *Hoxb7/Cre;Spry1*$^{flox/flox}$ mice were highly disorganized and exhibited multiple ureters, at a frequency (n = 11/15; Figure 4G) statistically similar to the kidneys of *Spry1*$^{-/-}$ animals (n = 20/21; Figure 2) (p = 0.14, Fisher's exact test). Therefore, we conclude that *Spry1* is required in the Wolffian duct to regulate kidney induction.

Spry1 Encodes an Antagonist of GDNF/RET Signaling in the Wolffian Duct

These results suggest a model in which *Spry1* regulates the site of UB outgrowth by antagonizing RET signaling in the Wolffian duct. To monitor GDNF/RET activity in the Wolffian duct, we assayed for *Wnt11* expression (Majumdar et al., 2003). Normally, *Wnt11* is detected only in UB tips, where there is a high level of RET activation (Majumdar et al., 2003). Significantly, in *Spry1*$^{-/-}$ embryos, *Wnt11* expression extended to more anterior positions along the Wolffian duct than in *Spry1*$^{+/-}$ embryos (Figures 5A and 5B). Discrete ectopic sites of *Wnt11* expression were also observed along *Spry1*$^{-/-}$ ducts (Figure 5B), presumably reflecting the sites where the supernumerary UBs form.

Although the mechanism(s) by which Sprouty proteins antagonize RTK signaling are incompletely understood, numerous studies indicate that they antagonize the RAS/MAPK pathway (Christofori, 2003; Kim and Bar-Sagi, 2004). If the normal function of SPRY1 in kid-

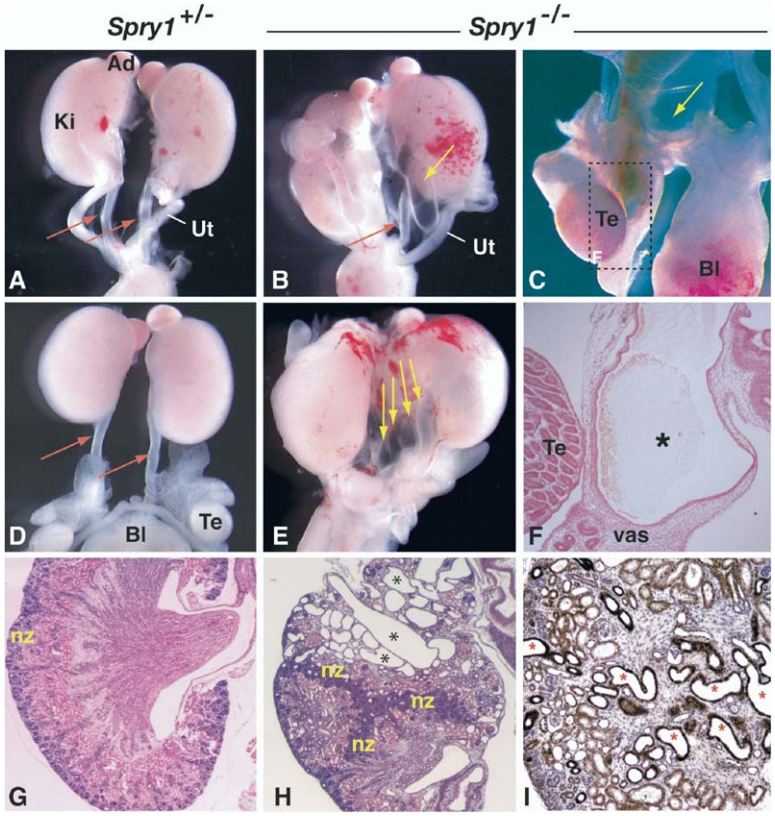

Figure 2. Kidney and Urogenital Tract Anomalies in *Spry1*$^{-/-}$ Mice

(A–E) Kidneys and urogenital systems from female (A and B) and male (C–E) *Spry1*$^{+/-}$ and *Spry1*$^{-/-}$ newborn pups are shown. Normal ureters and abnormal hydroureters are indicated by red and yellow arrows, respectively. A higher magnification of a hydroureter attached to the vas deferens in a male is shown (C).

(F) An H&E-stained section of the boxed area in (C) shows the hydroureter (asterisk) ending in the vas deferens.

(G–I) H&E-stained sections from control (G) and null (H) kidneys with nephrogenic zones indicated. Note the epithelial cysts (asterisks in [H]) that stain strongly with dolichus biflorus agglutinin (asterisks in [I]).

Abbreviations: Ad, adrenal; Bl, bladder; Ki, kidney; nz, nephrogenic zone; Te, testis; Ut, uterus; vas, vas deferens.

ney development is to antagonize RAS/MAPK signaling, one would expect to detect ectopic sites of MAPK activity in *Spry1*$^{-/-}$ Wolffian ducts. To investigate this, we assayed phosphorylated ERK (P-ERK) levels at ~E11 by immunohistochemistry. As suggested by a previous report (Fisher et al., 2001), high levels of P-ERK were present in the normal ureteric bud (Figure 5C). However, in *Spry1*$^{-/-}$ embryos, additional sites of P-ERK expression were detected in the ectopic, anterior UBs that formed along the Wolffian duct (Figure 5D), consistent with the hypothesis that the supernumerary UBs arise as a consequence of enhanced RTK activity.

These observations support the idea that *Spry1* prevents supernumerary UB formation by antagonizing GDNF/RET signaling in the posterior Wolffian duct. To provide a more direct test of this hypothesis, we compared the sensitivity of wild-type and *Spry1*$^{-/-}$ Wolffian ducts to GDNF. Affigel beads were soaked in 0.1–5 ng/μl GDNF and assessed for their ability to induce the formation of supernumerary ureteric buds in wild-type intermediate mesoderm explant cultures (Sainio et al., 1997; Tang et al., 2002). Beads soaked in <0.5 ng/μl GDNF were inefficient at inducing ectopic UBs from wild-type and *Spry1*$^{+/-}$ ducts, whereas beads soaked in GDNF at 0.75 ng/μl or higher always induced budding (n = 4/4). We reasoned that if the absence of *Spry1* rendered cells hypersensitive to GDNF, beads soaked in subthreshold concentrations of GDNF should induce a response in *Spry1*$^{-/-}$ Wolffian ducts. In accordance with this prediction, beads soaked in 0.5 ng/μl GDNF readily induced *Wnt11* expression in *Spry1*$^{-/-}$ Wolffian ducts (n = 4/5; Figure 5F), whereas exposure of *Spry1*$^{+/-}$ ducts to this low concentration of GDNF generally did not (n = 1/6; Figure 5E). Furthermore, beads soaked in 0.2 ng/μl GDNF elicited outgrowth of multiple UBs associated with PAX2-positive condensates from *Spry1*$^{-/-}$ Wolffian ducts (Figure 5H). By contrast, only occasional buds with weak or absent PAX2-positive condensates were induced in *Spry1*$^{+/-}$ ducts at this low concentration (Figure 5G). The most efficient UB induction occurred from the posterior Wolffian duct. Whereas a small number of buds were initiated from more anterior positions, no bud formation could be induced from most anterior, mesonephric regions of the Wolffian duct (not shown) in agreement with previous studies (Brophy et al., 2001). This suggests that the competence of the metanephric versus mesonephric duct to respond to GDNF has not been altered by the loss of *Spry1.* These results strongly support our hypothesis that *Spry1* regulates metanephric kidney induction by acting as a feedback antagonist of GDNF/RET signaling in the posterior Wolffian duct.

Reducing *Gdnf* Gene Dosage Rescues the *Spry1*$^{-/-}$ Phenotype

Our data suggested that the abnormal UB development and supernumerary bud formation in *Spry1*$^{-/-}$ embryos results from elevated sensitivity to GDNF signals. To definitively test this idea in vivo, we reduced the level of GDNF in *Spry1*$^{-/-}$ embryos by producing animals homozygous for the *Spry1* null allele and heterozygous for a *Gdnf* null allele (Sanchez et al., 1996). In only a

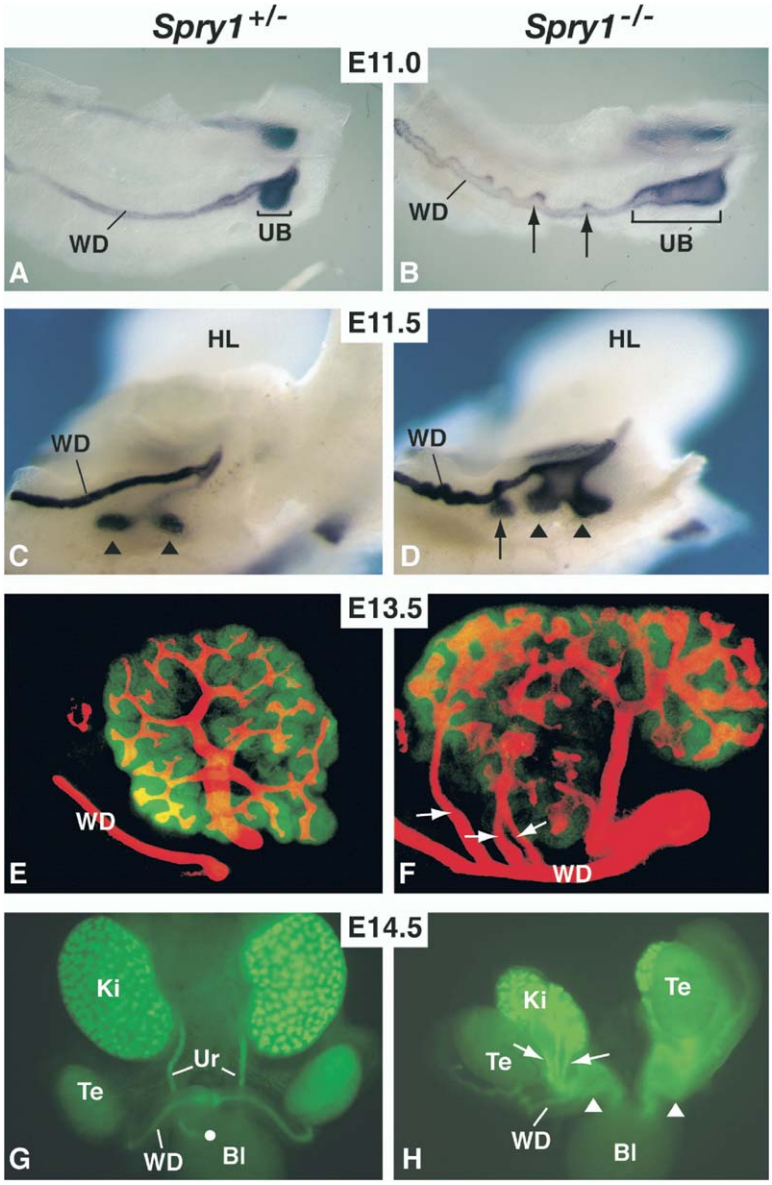

Figure 3. Abnormal Kidney Development in *Spry1*$^{-/-}$ Embryos

(A–D) Lateral views of intermediate mesoderm dissected from or exposed in *Spry1* heterozygous and null embryos, hybridized in whole mount with a probe for *Ret* RNA. Brackets indicate the ureteric buds (A and B). Note the abnormally wide UBs formed from *Spry1*$^{-/-}$ Wolffian ducts at E11.0. Arrows point to supernumerary UBs. Arrowheads point to the branched tips of the UBs at E11.5, which are significantly wider in the mutant embryos (C and D).

(E and F) E11.5 kidney explants cultured in vitro for 48 hr (equivalent to approximately E13.5) and stained for cytokeratin in red (ureteric tree) and PAX2 in green (condensing mesenchyme). Arrows indicate supernumerary ureteric trunks.

(G and H) Visualization of Wolffian duct derivatives (collecting ducts and developing ureters) in whole mount at E14.5 using a *Hoxb7/GFP* transgene (Srinivas et al., 1999a). In normal embryos, a single ureter from each kidney is joined to the bladder (white dot in [G] indicates one such connection). In *Spry1* null embryos, supernumerary ureters (arrows in [H]) are attached to the Wolffian duct, and dilated ducts (arrowheads) are found between the ureters and the bladder. Note also the abnormal position of the testes.

Abbreviations in addition to those used in Figure 2: HL, hindlimb bud; UB, ureteric bud; Ur, ureter; Wolffian duct, WD.

few of these *Spry1*$^{-/-}$;*Gdnf*$^{+/-}$ animals (4/16) was kidney development abnormal, whereas most of their *Spry1*$^{-/-}$;*Gdnf*$^{+/+}$ littermates (12/13) exhibited ureter and kidney abnormalities (Figures 5I–5K). Thus, reducing *Gdnf* dosage caused a striking 75% reduction in the incidence of CAKUT in *Spry1* null mice ($p = 0.0005$, Fisher's exact test). These data indicate that hyperresponsiveness of the Wolffian duct to GDNF is indeed a critical factor leading to the *Spry1* mutant phenotype.

Discussion

Recent studies suggested that the mammalian Sprouty genes have important roles in development and cancer by virtue of their ability to inhibit growth factor signaling (Kim and Bar-Sagi, 2004; Lo et al., 2004). Here, in our loss-of-function study to define the developmental function of a mammalian Sprouty gene, we provide compelling evidence that *Spry1* functions as a feedback antagonist of GDNF/RET signals during early kidney development. In particular, we show that *Ret* is required for the maintenance of high levels of *Spry1* expression in the Wolffian duct, which in turn serves to dampen the responsiveness of the duct to GDNF, thus preventing the formation of supernumerary UBs.

Sprouty Genes Antagonize RTK Signaling Pathways during Development

Although studies in *Drosophila* demonstrated that Sprouty inhibits signaling downstream of a number of RTKs, most reports to date have focused on the potential role of vertebrate Sprouty genes as negative regulators of the FGF signaling pathway during development, partly because the domains of expression of Sprouty family members are closely associated with sites of FGF activity during embryogenesis (Chambers and Ma-

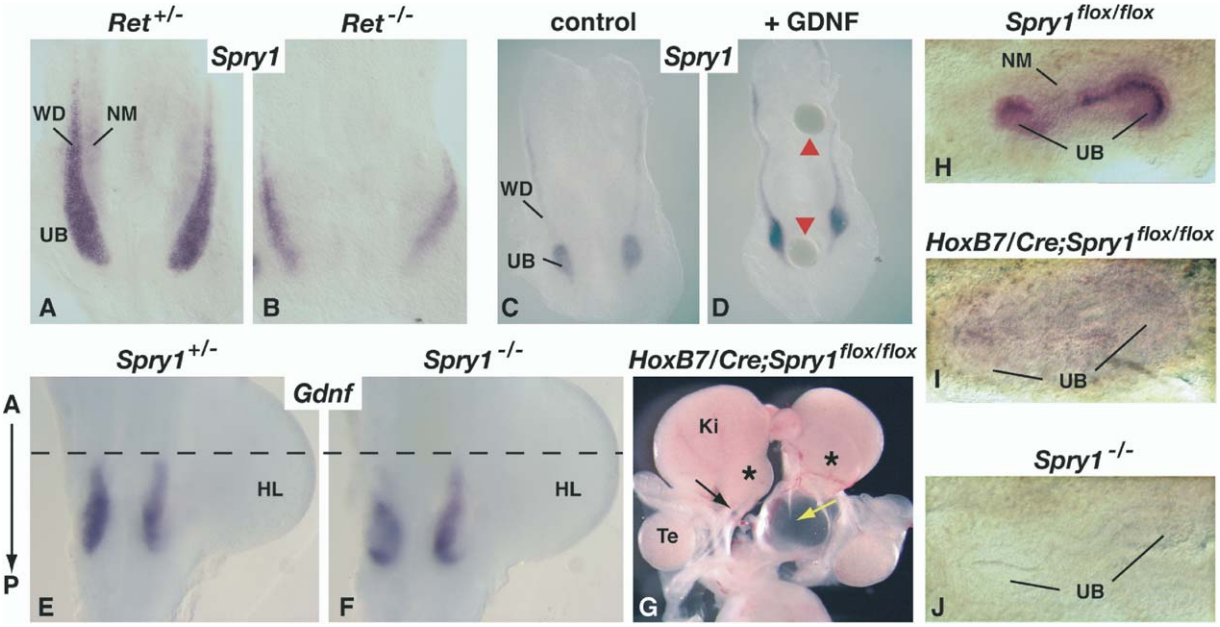

Figure 4. *Spry1* Gene Expression in the Wolffian Duct Is Regulated by GDNF/RET Signaling, where It Acts to Regulate Kidney Development

(A and B) Ventral views of intermediate mesoderm dissected from *Ret*$^{+/-}$ and *Ret*$^{-/-}$ embryos at E11.0, assayed in whole mount for *Spry1* expression. *Spry1* RNA is abundant in the Wolffian duct and presumptive UB and less abundant in the nephrogenic mesenchyme (NM) of a normal (*Ret*$^{+/-}$) embryo. *Spry1* expression is decreased in the Wolffian duct of a *Ret*$^{-/-}$ embryo.

(C and D) Ventral views of intermediate mesoderm dissected from E11.0 wild-type embryos assayed in whole mount for *Spry1* expression after culture for 2 hr in the absence (C) or presence (D) of GDNF-soaked beads (indicated by arrowheads).

(E and F) Ventral views of E11.0 (40–43 somites) *Spry1* heterozygous and null embryos assayed in whole mount for *Gdnf* expression. The anterior border of *Gdnf* expression domain is delineated by the broken line. In both genotypes, this border lies at a level approximately in the middle of the hindlimb bud.

(G) Kidneys and urogenital tracts from a *Hoxb7/Cre;Spry1*$^{flox/flox}$ newborn animal. Ectopic renal nodules (asterisks), a hydroureter (yellow arrow), and an extra ureter attached to the vas deferens (black arrow) are indicated.

(H–J) Lateral views of *Spry1*$^{flox/flox}$, *Hoxb7/Cre;Spry1*$^{flox/flox}$, and *Spry1*$^{-/-}$ intermediate mesoderm dissected at E11.5 and assayed for *Spry1* expression. In each panel, the tips of the UB branches are indicated. In the normal (*Spry1*$^{flox/flox}$) embryo, *Spry1* RNA is detected in both the UB epithelium and the nephrogenic mesenchyme (H). In the *Hoxb7/Cre;Spry1*$^{flox/flox}$ embryo, *Spry1* expression is specifically eliminated from the UB (I). No *Spry1* expression is detected in the *Spry1*$^{-/-}$ embryo (J).

son, 2000; de Maximy et al., 1999; Minowada et al., 1999; Zhang et al., 2001). Moreover, overexpression studies in cell culture consistently demonstrated that SPRY proteins inhibit FGF-mediated signaling (Gross et al., 2001; Hanafusa et al., 2002; Lim et al., 2000; Sasaki et al., 2001; Yusoff et al., 2002). In addition, overexpression or misexpression of Sprouty genes during development also implied that these genes inhibited FGF signaling (Chi et al., 2004; Furthauer et al., 2001; Mailleux et al., 2001; Minowada et al., 1999; Nutt et al., 2001; Perl et al., 2003; Tefft et al., 1999). For example, injection of *Xenopus Spry2* or *Drosophila spry* RNA into *Xenopus* oocytes inhibits the convergent extension movements during gastrulation, known to be dependent on FGF signaling (Nutt et al., 2001). While these studies pointed to a specific role for mammalian sprouty proteins in FGF-mediated signaling, cell culture studies showed that Sprouty family members can also inhibit signaling and biological effects induced by PDGF (Gross et al., 2001), HGF (Lee et al., 2004), VEGF (Impagnatiello et al., 2001; Lee et al., 2001; Sasaki et al., 2003), and NGF (Gross et al., 2001). Our genetic studies indicate that *Spry1* inhibits the GDNF/RET signaling pathway during kidney development. Thus, our data support the hypothesis that mammalian Sprouty genes, like their *Drosophila* ortholog, encode general RTK antagonists that can regulate developmental processes mediated by a variety of RTK signaling pathways. Our data do not rule out a role for Sprouty genes as inhibitors of FGF signaling during kidney development. In this regard, we note that a recent study demonstrated that expression of a human *SPRY2* transgene in the Wolffian duct affected the response of the duct to both FGFs and GDNF (Chi et al., 2004). Nevertheless, our finding that reducing *Gdnf* dosage rescues the *Spry1* phenotype indicates that an overactive response to GDNF is the critical factor responsible for supernumerary UB formation in *Spry1* null embryos.

Several Mechanisms Operate to Prevent Supernumerary Ureteric Bud Formation

The outgrowth of a single UB at a specific site along the Wolffian duct is imperative for normal kidney organogenesis. Gene targeting experiments identified several genes that are required to prevent ectopic UB formation. These include *Foxc1, Slit2*, and its receptor *Robo2,* which function to restrict *Gdnf* expression to the posterior mesenchyme at the onset of kidney de-

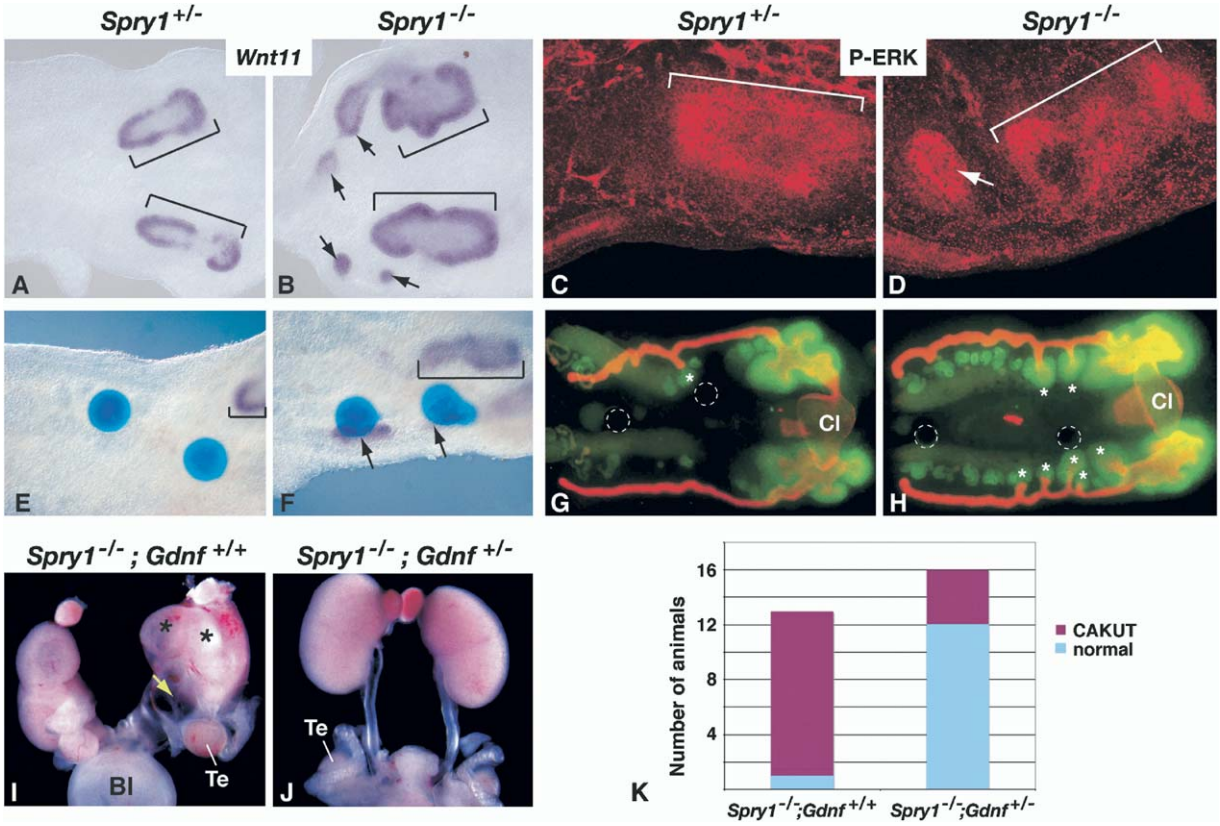

Figure 5. *Spry1*$^{-/-}$ Wolffian Ducts Are Hypersensitive to GDNF, and Reducing the *Gdnf* Gene Dosage Rescues the *Spry1*$^{-/-}$ Phenotype

(A–D) Ventral views of intermediate mesoderm from *Spry1* heterozygous and null embryos at ~E11.0.

(A and B) Hybridization in whole mount with a probe for *Wnt11* RNA. Expression is detected in the tips of UBs (brackets) and also in ectopic domains in the anterior Wolffian duct of the null mutant embryo (arrows in B).

(C and D) Immunostaining for active diphosphorylated ERK in the UB (brackets). Note the staining in an ectopic UB in the null mutant embryo (arrow in D).

(E and F) Affigel beads soaked in 0.5 ng/μl GDNF induce *Wnt11* expression in E10.5 *Spry1*$^{-/-}$ (arrows in F) but not in *Spry1*$^{+/-}$ (E) Wolffian ducts within 4 hr of culture. Brackets indicate *Wnt11* expression in UBs. Beads soaked in BSA alone did not induce ectopic *Wnt11* expression (not shown).

(G and H) Affigel beads soaked in 0.2 ng/μl GDNF (dotted circles) induce the formation of supernumerary ureteric buds (asterisks) with associated PAX2-positive mesenchymal condensates (green) from the Wolffian duct (red) in E10.5 *Spry1*$^{-/-}$ but not *Spry1*$^{+/-}$ embryos during 48 hr of culture. The cloacae (Cl) to which the posterior ducts attach are indicated.

(I and J) Newborn *Spry1*$^{-/-}$;*Gdnf*$^{+/+}$ and *Spry1*$^{-/-}$;*Gdnf*$^{+/-}$ kidneys and urogenital tracts are shown. Extrarenal nodules (asterisks) and hydroureter (arrow) are indicated.

(K) Bar graph showing the number of newborn mice displaying kidney and urinary tract defects (CAKUT). Mice were *Spry1* null and either wild-type or heterozygous for a null allele of *Gdnf.*

velopment, thus preventing activation of RET signaling at more anterior positions along the Wolffian duct (Grieshammer et al., 2004; Kume et al., 2000). In contrast, loss of *Spry1* function does not result in persistence of *Gdnf* expression in the anterior mesenchyme. This observation, coupled with our finding that deletion of *Spry1* specifically in the Wolffian duct causes the same phenotype as is observed in *Spry1* null embryos, supports the hypothesis that *Spry1* regulates signaling downstream of RET by rendering the Wolffian duct less sensitive to GDNF. In support of this model, we demonstrate that inactivation of *Spry1* results in hypersensitivity of the Wolffian duct to GDNF protein. These data indicate that tight control of *Gdnf* expression alone is not sufficient to prevent supernumerary UB formation and that negative feedback regulation of RET activation in the Wolffian duct by *Spry1* is critical to ensure normal kidney induction.

An alternative explanation for our results might be that loss of *Spry1* function causes hyperactivation of an RTK other than RET, which in turn upregulates *Ret* expression, thereby increasing RET signaling and UB formation. However, this seems unlikely for several reasons. First, we found no significant difference in *Ret* expression at E11 between control and *Spry1*$^{-/-}$ Wolffian ducts as measured by quantitative PCR (data not shown). Second, in prior studies, overexpression of *Ret* in the Wolffian duct did not lead to ectopic UB formation unless *Ret* was mutated to a ligand-independent form (de Graaff et al., 2001; Srinivas et al., 1999b). These data are consistent with our conclusion that increased RET signaling in the absence of SPRY1 causes the for-

mation of supernumerary UBs and suggest that a search for genes responsible for human urinary tract anomalies should include those that encode negative regulators of GDNF/RET activity such as *Spry1*.

Signaling Pathways Regulated by SPRY1 during Kidney Induction

The mechanism by which Sprouty genes exert their effects remains controversial (Christofori, 2003; Kim and Bar-Sagi, 2004). In some cultured cells overexpressing Sprouty genes, enhanced signaling was reported, challenging the notion that Sprouty proteins act only as antagonists (Christofori, 2003; Egan et al., 2002; Guy et al., 2003; Rubin et al., 2003; Tefft et al., 1999). However, our data support a model in which SPRY1 serves as a feedback antagonist of GDNF/RET signaling during renal organogenesis.

Numerous studies have suggested that Sprouty proteins function by inhibiting the RAS/MAPK pathway. Our finding that the ectopic UBs in *Spry1*$^{-/-}$ embryos contain high levels of P-ERK support the hypothesis that SPRY1 affects the RAS/MAPK pathway. However, it has been reported that GDNF-induced outgrowth of ectopic UBs from the Wolffian duct could be prevented by pharmacological inhibition of the PI3K pathway but not the RAS/MAPK pathway (Tang et al., 2002). Moreover, *Xenopus* SPRY2 interferes with Ca^{2+} signaling during development (Nutt et al., 2001). Thus, it remains to be determined what signaling pathways downstream of RET are responsible for UB formation and which of these are regulated by SPRY1.

Mammalian Sprouty Genes Are Coexpressed during Kidney Induction

At many stages of development, *Spry1, Spry2*, and *Spry4* are coexpressed in at least partially overlapping domains (de Maximy et al., 1999; Minowada et al., 1999; Zhang et al., 2001), raising the possibility that these genes are functionally redundant. At the onset of kidney development, both *Spry1* and *Spry2* are expressed in the Wolffian duct (M.A.B., unpublished data). However, mice lacking the *Spry2* gene do not exhibit any defects in kidney induction (U. Grieshammer, M.A.B., G. Minowada, and G.R.M, unpublished data). Hence, these Sprouty genes might perform different functions during kidney development. Alternatively, they may both contribute to the regulation of GDNF/RET signaling, but *Spry1* activity may be sufficient to support normal kidney development in the absence of *Spry2*. This type of functional redundancy has been observed for FGF genes in the developing limb bud: although inactivation of *Fgf4* has no effect on its own (Moon et al., 2000; Sun et al., 2000) and inactivation of *Fgf8* causes only a mild limb phenotype (Lewandoski et al., 2000; Moon and Capecchi, 2000), inactivation of both genes in the same domain causes complete failure of limb development (Sun et al., 2002). Thus, one might predict an exacerbation of the UB phenotype in *Spry1*;*Spry2* double null mutants. Such genetic studies may clarify the potential redundancy of Sprouty genes and further elucidate the essential functions of these negative feedback inhibitors in development.

Experimental Procedures

Construction of the *Spry1* Targeting Vector

A 129/Sv mouse genomic BAC library was screened by PCR, and two clones containing the Sprouty1 gene were identified (Incyte Genomics, Palo Alto, CA). An 11.5 kb EcoRI fragment that contains all three putative exons, including the third exon that encodes the complete open reading frame (ORF), was subcloned into pBluescript. Oligonucleotides containing *loxP* sequences were inserted into *Bgl*II sites flanking the ORF. Correct insertion and orientation were confirmed by sequencing. A PGKneo cassette flanked by *frt* sequences was inserted into a unique *Bcl*I site 3′ of the *Spry1* ORF to generate the targeting construct.

Analysis of Homologous Recombination

The targeting vector, linearized by *Not*I digestion, was electroporated into R1 ES cells (A. Nagy, Toronto). G418-resistant ES cell colonies were isolated and Southern blotting was performed on genomic DNA digested with *Xba*I or *Spe*I, using a fragment located 5′ of the sequences in the targeting construct as a probe. Eight out of a total of 196 clones had undergone homologous recombination, indicated by a 7.8 kb fragment in *Xba*I digests and a 12 kb band in SpeI digests. Two clones were injected into C57BL/6 blastocysts (K. Kelley, Mouse Genetics Shared Research Facility, Mount Sinai). Chimeric males were mated with C57BL/6J females, and pups derived from the 129/SvJ ES clones were screened for the presence of the targeted allele by PCR of genomic DNA using the primers described below.

Generation of Mouse Strains and Genotyping by PCR

Mice carrying the targeted allele were bred with Flpe deleter mice (Rodriguez et al., 2000) to remove the PGK-neo cassette. Mice carrying *Spry1*^{flox} were crossed with *β-Actin/Cre* deleter mice (Lewandoski et al., 1997) on an FVB/N genetic background to remove the *Spry1* ORF and generate a null allele. PCR genotyping confirmed deletion of the ORF (Figure 1). All subsequent analyses were performed in this mixed genetic background (129Sv/J; C57BL/6; FVB/N). Animals carrying a null allele in which the neo cassette was not deleted were generated by crossing chimeric males directly with *β-Actin/Cre* females. Animals homozygous for this null allele and thus on a 129Sv/J; FVB/N genetic background exhibited a kidney phenotype similar to the one described.

The primers used to genotype these animals by PCR were: P1: CTCAATAGGAGTGGACTGTGAAACTGC; P2: GGGAAAACCGTGTT CTAAGGAGTAGC; P3: GTTCTTTGTGGCAGACACTCTTCATTC.

Tissue-specific knockouts were generated by breeding *Spry1*$^{flox/flox}$ females to males carrying a *Hoxb7/Cre* transgene (Yu et al., 2002) and heterozygous for *Spry1*^{flox} (*Hoxb7/Cre;Spry1*$^{flox/+}$). Mice carrying a *Gdnf* null allele (Sanchez et al., 1996) on a C57BL/6 background were crossed to *Spry1*$^{+/-}$ animals. *Spry1*$^{+/-}$*; Gdnf*$^{+/-}$ animals were intercrossed to produce *Spry1*$^{-/-}$*;Gdnf*$^{+/-}$ and *Spry1*$^{-/-}$*;Gdnf*$^{+/+}$ newborns, which were analyzed for defects in kidney development. All mouse experiments were approved by the Mount Sinai Institutional Animal Care and Use Committee (USA) and the Home Office (UK).

Analysis of Gene Expression by RT-PCR

Total RNA was isolated from fresh tissue using RNA-Stat 60 (Tel-Test Inc., Friendswood, TX). RT-PCR analyses were performed using the Promega RT-PCR system. Primers were designed to amplify 300 bp fragments from the *Spry1* and *Spry2* coding regions. Reactions without reverse transcriptase (RT) served as controls for genomic DNA contamination.

Whole-Mount In Situ Hybridization

Intermediate mesoderm from E10.5–E11.5 embryos was isolated and fixed overnight in 4% paraformaldehyde in PBS (PFA) at 4°C. In situ hybridization was carried out using probes for *Ret* (Srinivas et al., 1999b), *Wnt11* (Majumdar et al., 2003), and *Spry1* and *Spry2* (Minowada et al., 1999). A combination of two probes were used to

detect *Gdnf*: one described by Srinivas et al. (1999b) and a shorter probe provided by U. Grieshammer (UCSF, San Francisco, CA).

Histological Analysis

Newborn mice were sacrificed according to institutional and national guidelines. Whole kidneys and urogenital tracts were dissected and images captured under a Leica MZAPO microscope attached to a SONY-DXC970MD digital camera using Scion Image software. The tissues were fixed for a few hours to overnight in 4% PFA at 4°C, embedded in paraffin, and 5 μm sections were cut and stained with hematoxylin and eosin (H&E).

Intermediate Mesoderm and Metanephric Kidney Explant Cultures

Intermediate mesoderm or metanephric kidneys were dissected from E10.5 or E11.5 embryos, respectively, in ice-cold CO_2-independent medium (GIBCO) containing 1% fetal bovine serum (GIBCO). Explants were cultured at 37°C in DMEM (GIBCO) supplemented with 290 μg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO_2, humidified atmosphere at the medium-air interface on Costar Transwell filters (0.4 μm). For bead experiments, Affigel blue beads (100–200 mesh, Biorad, Hemel Hempstead, UK) were soaked in recombinant human GDNF (Promega, Southampton, UK) at the indicated concentration for 1 hr at room temperature, rinsed in PBS, and applied to the explant culture. After culture, explants were fixed either in methanol for immunostaining or 4% PFA for in situ hybridization. For immunostaining, explants were incubated with murine anti-cytokeratin (Sigma, Surrey, UK) and rabbit anti-PAX2 (Covance, Cambridge Bioscience, Cambridge, UK) antibodies, followed by Alexa488 anti-mouse Ig and Alexa546 anti-rabbit Ig (Molecular Probes, Invitrogen, Paisley, UK). Staining for P-ERK was performed as described (Corson et al., 2003). Images were captured on a Zeiss axioskop microscope attached to a Colorview12 digital camera.

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